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Lymphocyte mitogenesis induced by a mammalian liver protein that specifically binds desialylated glycoproteins

[thymus-derived (T)-lymphocytes/hepatic binding protein/asialoglycoproteins/neuraminidase]

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ABSTRACT **A purified rabbit liver membrane protein that binds desialylated glycoproteins has been shown to be a mitogen for human peripheral lymphocytes. The mitogenic activity is specific for desialylated thymus-derived (T)-cells. The loss of mitogenicity upon exposure of the binding protein to neuraminidase and the inhibitory potency of asialo-orosomucoid support the conclusion that the site involved in the binding of asialoglycoproteins is also responsible for the mitogenic effect on desialylated lymphocytes. The potential relevance of this phenomenon to the etiology of hepatocellular necrosis is considered.**

Mitogens induce lymphocyte transformation by reacting with specific carbohydrate residues on the surface membrane of the cell (1, 2). The target sites for mitogens such as phytohemagglutinin (PHA), concanavalin A (Con A), or periodate are fully exposed on the cell surface, whereas the sites for soybean agglutinin (SBA), peanut agglutinin (PNA), and galactose oxidase are masked by sialic acid residues and require treatment with neuraminidase prior to activation. The penultimate galactose residues, exposed by the enzymatic release of sialic acid, are presumed to be the target site for the latter mitogens (3–5).

The isolation and characterization of a rabbit liver binding protein specific for galactose-terminated glycoproteins has been described (6, 7) and the protein has been shown to possess the lectin-like ability to agglutinate erythrocytes (8). In some species, agglutination was made manifest only after neuraminidase treatment of the cells.

The present report extends these findings with the demonstration that this hepatic binding protein (HBP) is a mitogen. The data indicate that the binding site involved in the induction of mitogenesis in desialylated lymphocytes is similar or identical to that responsible for the binding of desialylated glycoproteins.

MATERIALS AND METHODS

Soybean and peanut agglutinin purified by affinity chromatography (9, 10) were kindly supplied by R. Lotan and N. Sharon of the Weizmann Institute, Rehovot, Israel. Twice-crystallized concanavalin A was purchased from Miles-Yeda, Ltd. and neuraminidase from Grand Island Biological Co. of New York. The latter was obtained as a solution containing 500 units/ml. One unit is defined as the amount of enzyme capable of cleaving 1 μg of sialic acid from α_1-acid glycoprotein in 15 min at 37° and at pH 5.5. [*methyl*-^{3}H] Thymidine (2 Ci/mmol) was obtained from New England Nuclear Co. Orosomucoid (α_1-acid glycoprotein) was isolated from pooled human plasma and desialylated as described previously (11). The hepatic protein, purified to homogeneity from whole rabbit liver by affinity chromatography (7), had a specific binding activity of 40 μg of asialo-orosomucoid per mg of binding protein.

Isolation of Cells. Circulating mononuclear cells were obtained from the peripheral blood of normal human subjects by Ficoll-Hypaque gradient centrifugation (12), and contained 70–90% lymphocytes, 10–30% monocytes, and 1–3% granulocytes. Adherent cells [monocytes and adherent bone-marrow-derived (B)-cells] were removed by the following procedures: (*a*) Unfractionated cells suspended in RPMI 1640 medium containing 20% heat-inactivated fetal calf serum were added to a 30 ml plastic syringe containing nylon wool. After incubation for 60 min at 37° the nonadhering cells, now enriched to 95–99% lymphocytes, were collected by elution with phosphate-buffered saline, pH 7.2. (*b*) Unfractionated cells (5 ml, 2×10^6/ml) suspended in the above medium were incubated in plastic tissue culture petri dishes (Falcon 3003, 60 × 15 mm) for 60 min at 37° and the nonadherent cells were harvested.

Fractionation of Lymphocytes. Lymphocytes described above were fractionated by the sheep red blood cell rosetting method (13). Lymphocytes forming or not forming rosettes with sheep erythrocytes are referred to as thymus-derived (T)-lymphocytes or B-lymphocytes, respectively.

Lymphocyte Cultures. Lymphocytes (1×10^6/ml), suspended in RPMI 1640 medium containing heat-inactivated fetal calf serum (5%) and supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), were cultured (0.2 ml aliquots) in flat bottom microwells (Microtest II, Falcon 3040) at 37° in a 95% air/5% CO_2 atmosphere for 72 hr. At 20 hr prior to the termination of the incubation, 2 μCi of [*methyl*-^{3}H]thymidine (in 50 μl of medium) was added to each culture well and incorporation into DNA was measured at the end of the incubation period (14). The results, as cpm, are expressed as the mean of duplicate cultures.

Mitogenic Stimulation of Cells. Unless otherwise specified, cells were exposed to the various mitogens at the following final concentrations (μg/ml): Con A, 2; soybean agglutinin, 50; peanut agglutinin, 125; hepatic binding protein, 50.

Treatment of Cells with Neuraminidase. Cells (10 to 20 × 10^6/ml) in phosphate-buffered saline were treated with neuraminidase (50 units/ml) at 37° for 30 min with shaking followed by two washings with the buffered saline to remove excess reagent prior to suspension in culture medium.

RESULTS

A comparison of the mitogenic effect of several lectins on intact and desialylated human peripheral lymphocytes is provided in Table 1. The hepatic binding protein stimulates neuraminidase-treated lymphocytes and is essentially inert towards the

Abbreviations: Con A, concanavalin A; PNA, peanut agglutinin; SBA, soybean agglutinin; HBP, hepatic binding protein; B-cell, bone-marrow derived cell; T-cell, thymus-derived cell.

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Table 1. Stimulation of unfractionated human peripheral lymphocytes by hepatic binding protein and phytomitogens

Treatment		[^{3}H]Thymidine incorporation (cpm) in subjects					
Mitogen	Neuraminidase	1	2	3	4–1*	4–2*	4–3*
—	—	550	630	185	910	2,300	470
—	+	735	500	300	570	1,130	440
HBP	—	430	1,470	215	830	1,820	540
HBP	+	3,755	3,140	2,260	2,950	10,630	3,480
SBA	—	845	670	580	2,610	9,750	3,000
SBA	+	5,175	3,020	16,130	21,490	44,970	23,410
PNA	—	510	900		670		695
PNA	+	5,110	3,660		1,755		6,900
Con A	—		28,300		29,570		38,315
Con A	+		35,000		30,380		23,410

Experimental conditions are given in *Materials and Methods.*
* The same subject was tested on three different dates.

intact cells, a behavior pattern indistinguishable from that seen with peanut agglutinin. In contrast, the mitogenic effect of Con A is maximal and invariant with respect to the external sialic acid complement of these cells, whereas soybean agglutinin has an intermediate effect and attains full stimulatory activity only after enzymatic exposure of the underlying galactose residues. A marked increase in the agglutinability of the neuraminidase-treated lymphocytes was noted in the presence of the hepatic binding protein; this effect was not observed on the untreated cells.

Recent studies (15) have shown that the removal of adherent cells from unfractionated lymphocyte preparations enhances the response of the remaining lymphocytes to soybean and peanut agglutinins significantly. As shown in Table 2, the response to the hepatic binding protein is similarly enhanced and the response is maximal only after neuraminidase treatment of the cells. The mammalian protein exhibited a selective stimulatory effect on T-cells (Table 3), a specificity previously demonstrated for both soybean and peanut agglutinin (15). Maximal stimulation occurred at a concentration of 50 μg/ml of the hepatic binding protein; increasing levels were inhibitory and the resulting bell-shaped curve (Fig. 1) is similar to that recorded previously for other lectins.

Earlier studies have shown the integrity of the terminal sialic acid residues of the hepatic binding protein to be an absolute requirement for binding asialoglycoproteins (6) and for the agglutination of erythrocytes (8). Table 4 illustrates an identical requirement for mitogenicity. Blastogenic activity of the binding protein was abolished by neuraminidase; Con A was unaffected. A similar result was produced by competitive inhibition with an appropriate binding substrate. Thus, the addition of 50 μg/ml of asialo-orosomucoid to the reaction mixture totally inhibited the mitogenic activity of the binding protein; this effect was not observed upon addition of the intact orosomucoid protein.

DISCUSSION

A considerable body of evidence has been accumulated to support the view that the hepatic binding protein used in these studies plays a significant role in the catabolism of desialylated serum glycoproteins (16). The recent observation that it also possesses lectin-like properties in its ability to agglutinate erythrocytes (8) raised the possibility that it might mimic the plant lectins, peanut and soybean agglutinins, by inducing mitosis in desialylated human lymphocytes. The data recorded here provide unequivocal evidence that this mammalian protein is a potent mitogen that selectively stimulates T-cells. The loss of mitogenicity upon exposure to neuraminidase (Table 4) and the inhibitory potency of asialo-orosomucoid support the

Table 2. Effect of various mitogens on human peripheral lymphocytes depleted of adherent cells

Treatment		[^{3}H]Thymidine incorporation (cpm)					
		Experiment 1			Experiment 2		
Mitogen	Neuraminidase	Unfractionated	Column-purified*	Petri-dish-purified*	Unfractionated	Column-purified*	Petri-dish-purified*
—	—	630	2,090	3,315	1,570	1,890	1,765
—	+	500	3,900	4,070	2,310	2,820	1,890
HBP	—	1,470	6,050	5,600	1,480	1,860	2,820
HBP	+	3,140	23,620	23,820	9,230	20,325	32,550
SBA	—	670	3,225	4,195			
SBA	+	3,020	15,020	22,890			
PNA	—	895	2,070	8,990	1,970	1,840	2,050
PNA	+	3,660	28,040	49,430	11,730	21,920	23,610
Con A	—	28,310	45,990	49,300	53,680	46,920	36,740
Con A	+	34,988	50,080	84,590	47,310	56,920	72,940

* As described in *Materials and Methods.*

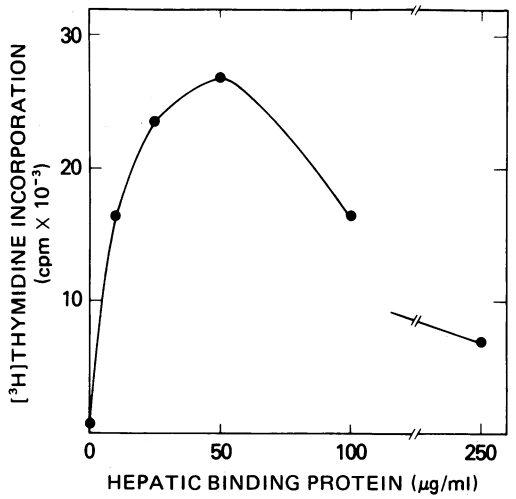

FIG. 1. Response of human peripheral lymphocytes to increasing concentrations of the hepatic binding protein. Lymphocytes, purified by the column procedures, were desialylated and assayed as described in *Materials and Methods*.

conclusion that the site involved in the binding of asialoglycoproteins is also responsible for the triggering of lymphocytes. Thus, we report here a mammalian protein, with the carbohydrate binding properties of a lectin, that has mitogenic activity.

In an earlier report, Woodruff and Gesner (17) demonstrated that neuraminidase-treated lymphocytes accumulated in the liver and the possibility should be considered that such lymphocytes are removed from the circulation by a mechanism similar to that described for desialylated glycoproteins (16).

Of more immediate pertinence, however, are the very recent studies of Woodruff and Woodruff (18, 19) on the interaction of influenza virus and lymphocytes. After brief incubation of thoracic duct lymphocytes with influenza virus at 37°, sialic acid was liberated from the cell surface as a consequence of the viral neuraminidase. Upon subsequent infusion of these lymphocytes into syngeneic recipients, the lymphocytes accumulated predominantly in the liver with their recovery in the lymph nodes and spleen being severely reduced. These observations lend strong support to the hypothesis that infection by myxoviruses, or by any agent exhibiting neuraminidase activity, may result in the hepatic entrapment of desialylated lymphocytes. In view of the known potential of mitogen-stimulated lymphocytes for cytotoxicity (20), the binding protein, present on the plasma membrane of hepatocytes (21), appears to be a possible factor in the onset of hepatocellular necrosis of unknown etiology.

Table 3. Response of T and B human peripheral lymphocytes to HBP and phytomitogens

Treatment		[³H]Thymidine incorporation (cpm)		
Mitogen	Neuraminidase	Unfractionated cells	T-cells	B-cells
—	—	470	100	240
—	+	440	100	310
HBP	—	540	140	500
HBP	+	3,470	35,170	1,090
SBA	—	3,000	6,730	700
SBA	+	23,410	58,880	1,755
PNA	—	695	120	670
PNA	+	6,890	31,470	1,050
Con A	—	38,315	52,490	2,100
Con A	+	25,220	35,170	2,640

Table 4. Effect of neuraminidase on HBP and Con A

Mitogen	Neuraminidase treatment of mitogen	[³H]Thymidine incorporation (cpm)
—	—	1,370
HBP	—	14,020
HBP	+	1,520
Con A	—	60,390
Con A	+	57,800

The hepatic binding protein (1 mg/ml) and concanavalin A (0.04 mg/ml) were incubated with neuraminidase (final concentration, 50 units/ml) for 30 min at 37°. Aliquots of 10 μl were added to the appropriate cell cultures in a total volume of 0.2 ml and the assay was carried out as described in *Materials and Methods*.

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